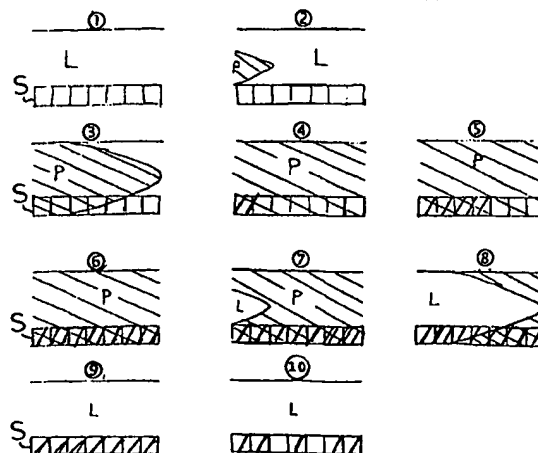
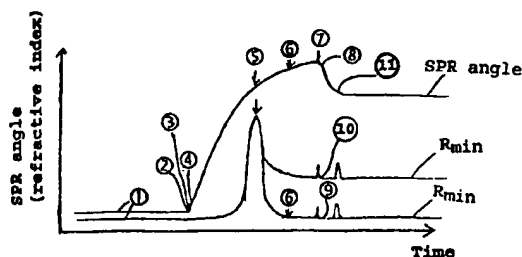




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(21) International Application Number: PCT/SE96/01074 (22) International Filing Date: 30 August 1996 (30.08.96) (30) Priority Data: 9503028-4 1 September 1995 (01.09.95) SE (71) Applicant (for all designated States except US): PHARMACIA BIOSENSOR AB [SE/SE]; S-751 82 Uppsala (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): IVARSSON, Bengt [SE/SE]; Fögderivägen 22, S-740 22 Bålinge (SE). STENBERG, Esa [SE/SE]; Folkungagatan 20B, S-753 36 Uppsala (SE). (74) Agents: WIDÉN, Björn et al.; Pharmacia & Upjohn AB, Patent Dept., S-751 82 Uppsala (SE).		(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: MONITORING OF REFRACTIVE INDEX OF OPTICAL SENSOR FOR DETERMINATION OF SURFACE STRUCTURE CHANGES		
(57) Abstract <p>The invention relates to a method of analysing a chemical or physical interaction taking place in a film layer at an optical sensor surface when the film layer is contacted with a fluid sample containing a species capable of interacting with the film layer, wherein the interaction is monitored by determination of the refractive index of said film layer through a light-intensity signal producing technique by measuring the relationship between a parameter of the incident and/or reflected light and one of the minimum, the maximum and the centroid of the light intensity signal curve. The method is characterized by also monitoring the variation of the light intensity signal value at said minimum, maximum or centroid with time to determine at least one of (i) the degree of homogeneity of sample species concentration in the sensed film layer volume to determine therefrom when the interaction is mass transport limited and when the interaction is kinetically controlled, a varying light intensity signal level indicating mass transport limitation and a stabilized light intensity signal level indicating kinetic control; and (ii) the degree of resulting homogeneity/heterogeneity in the sensed film layer volume during or after the interaction, a changed stabilized light intensity signal level indicating and corresponding to the degree of a change in homogeneity/heterogeneity.</p>		



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Monitoring of refractive index of optical sensor for determination of surface structure changes.

FIELD OF THE INVENTION

5 The present invention relates to an improved method of analysing physical or chemical interactions taking place in a film layer at an optical sensor surface by means of refractive index determination through light intensity signal producing techniques.

BACKGROUND OF THE INVENTION

10 Recently, the interest for surface sensitive measuring techniques has increased markedly as several so-called label-free optical techniques have been developed for measuring and quantifying biomolecular interactions. A so
15 far frequently used such optical technique is based on surface plasmon resonance, hereinafter often referred to as SPR.

 When light travels from an optically denser medium (i.e. having a higher refractive index) to a less dense
20 medium (i.e. having a lower refractive index), total internal reflection (TIR) occurs at the interface between the two media if the angle at which the light meets the interface is above a critical angle. When TIR occurs, an electromagnetic "evanescent wave" propagates away from the
25 interface into the lower refractive index medium.

 If the interface is coated with a thin layer of certain conducting materials (e.g. gold or silver), the evanescent wave may couple with free electron constellations, called surface plasmons, at the conductor
30 surface. Such a resonant coupling occurs at a specific angle of the incident light, absorbing the light energy and causing a characteristic drop in the reflected light intensity at that angle. The surface electromagnetic wave creates a second evanescent wave with an enhanced electric
35 field penetrating into the less dense medium

 The resonance angle is sensitive to a number of factors including the wavelength of the incident light and the nature and the thickness of the conducting film. Most

importantly, however, the angle depends on the refractive index of the medium into which the evanescent wave of the surface plasmon wave propagates. When other factors are kept constant, the resonance angle is thus a direct measure of the refractive index of the less dense medium, the angle being very sensitive to refractive index changes in the medium. For a detailed description of the dielectric equations describing this dependence it may be referred to Kretschmann, E., Z. Phys. B241, 313 (1971).

10 The SPR evanescent wave decays exponentially with distance from the interface, and effectively penetrates the lower refractive index medium to a depth of approximately one wavelength. Therefore, only changes in refractive index very close to the interface may be detected.

15 If the metal film is covered with an appropriate sensing layer (e.g. an antibody) capable of specific interaction with a molecule (e.g. an antigen) present in a fluid sample contacted with the sensing layer, SPR based chemical sensors may be constructed, the interaction at the sensor surface changing the solute concentration and bound surface concentration and thus the refractive index within the penetration range of the evanescent wave. A variety of SPR-based chemical sensors have been developed wherein the change of the reflectance curve (reflected light intensity versus angle of incidence or wavelength) with time is measured, which change is correlated to the surface refractive index.

20 Different techniques may be used for bringing the light to interact with the sensor surface. A commonly used detection system is based on the Kretschmann configuration (Kretschmann and Raether, Z. Naturforsch. Teil A 23: 2135-2136, 1968). In this configuration a thin layer of the reflective metal (gold or silver) is deposited on the base of a prism and TM polarized, monochromatic light is coupled by the prism to the SPR-wave.

35 An example of a commercial biosensor system constructed on the basis of the Kretschmann configuration is BIAcore™, marketed by Pharmacia Biosensor AB (Uppsala,

Sweden). This biosensor system incorporates a Kretschmann configuration-based SPR-detection system with a microfluidic system to control the flow of reagents required in the analyses. With this apparatus, biomolecular interactions occurring at the sensor surface may be monitored in real time. The apparatus and theoretical background thereof has been fully described by Jönsson et al., 1991, BioTechniques 11, 620-627. It is also referred to our US-A-5,313,264.

When monitoring the biomolecular interactions, the development of the SPR-response, or surface film refractive index, with time is followed. From this relationship not only the biomolecule analyte concentration may then be determined but also kinetic parameters, such as association and dissociation rate constants for the interaction of the biomolecule with the sensor surface.

As an alternative to the reflectance minimum angle, the angle of the centroid of the reflectance curve may be monitored.

Rather than measuring the incident light angle where SPR (i.e. reflectance minimum) occurs, some SPR sensors, as already mentioned above, introduce the light at a fixed angle and measure the wavelength at which SPR takes place (see e.g. US-A-5,359,681), SPR in both cases, however, representing a reflectance minimum of the reflectance curve.

When studying kinetic parameters, it is, of course, important to know when the interaction at the surface is mass transport limited and when it is reaction-kinetically controlled. This may not readily be determined from the above-mentioned refractive index versus time curve, and there is therefore always a risk of an incorrect estimate of kinetic constants. Further, it may not be readily determined from the refractive index versus time curve whether the interaction at the surface has caused any structural change of the surface resulting in a heterogeneity.

In other contexts than the chemical SPR sensors, parameters such as the intensity of the reflectance minimum (R_{\min}) and the halfwidth ($\Phi_{1/2}$) of the reflectance curve have been studied.

5 Pockrand, I., Surface Science, vol. 72, p. 577-588 (1978) describes the influence of thin dielectric coatings on the properties of surface plasma oscillations propagating along a metal surface using an attenuated total reflection (ATR) arrangement to excite
10 SPR. A general shift and a broadening of the resonance curve are observed. The depth of the resonance R_{\min} is unaffected by transparent coatings, whereas it is strongly dependent on the thickness of an absorbing coating.

15 Fontana, E., Pantell and Moslehi, Applied Optics, vol. 27, (1988) p. 3336 characterizes dielectric-coated metal mirrors using surface plasmon spectroscopy. Analytical expressions for SPR angle shift, halfwidth and reflectivity minimum are described.

20 Chu, K.C., et al., Mol. Cryst. Liq. Cryst., vol. 59, p. 97-108, (1980) discloses a study of isotropic-nematic phase transition of 4-cyano-4'-n-pentylbiphenyl on gold as a function of temperature by surface plasmon resonance technique. Changes in reflectance curve
25 halfwidth and the occurrence of two reflective dips were observed. One of the two resonant angles corresponds to the refractive index of the isotropic phase, and the other to the refractive index of the nematic phase. The relationship between the dip depths
30 of the "double dip reflectivity curve" was used as a measure of the fraction of the medium in each of the two phases.

Pollard, J.D., and Sambles, J.R., Optics Communications, vol. 64, p. 529-533 (1987) describes
35 the analysis of the time-dependence of the SPR reflectance curve parameters, reflectance minimum angle, reflectance minimum depth, and reflectance curve width to study the lateral extension of the two phases

of a condensed liquid, i.e. as droplets and as a homogeneous film, respectively. The existence of two distinct refractive indices in different parts of an illuminated gold surface gave two reflectance curves which together gave a reflectance curve with two minima, the depth of the respective minimum corresponding to the mutual degree of coverage of the gold surface.

Rothenhäusler and Knoll, Surface Science, vol. 191, p. 585-594, (1987) discloses reflectivity versus angle scans in the Kretschmann ATR configuration of an silver-air interface with p-polarized laser light. If there is within the area of the laser spot on the sample film a step from one thickness to another, two resonance minima are obtained, each at an angle corresponding to one of the two "infinite" layer structures. The intensities of the resonance minima depend on the relative area fraction of the two different interfaces covered by the laser spot.

Rothenhäusler and Knoll, Appl. Phys. Lett., vol. 51, p. 783-785, (1987) discloses the use of SPR in combination with diffraction to study a discrete film of two media with different layer structures. The mutual relationship between the reflectance minima of two simultaneous SPR's is used to determine the degree of surface coverage for the respective media.

Yeatman and Ash, SPIE, vol. 897, p. 100-107, (1988) p. 107 discloses a technique for SPR microscopy for studying lateral structure (surface heterogeneity distribution) in biological monolayers and other superimposed layers. It is proposed that the position, width and depth of the reflectance minimum be measured to permit different contrast mechanisms to be separable due to their different relative effects on the three parameters.

Zhang, Y. et al, Surface Sci., vol. 184, p. 214-226, (1987) describes the use of surface plasmon oscillations for the investigations of the kinetics of

adsorption of a polystyrene film onto a metallic surface. The resonance angular shift, the halfwidth of the dip and the value of the resonance minimum is used for determining the characteristics of the adsorbed layer.

Silin, V. I., et al., Optics Communications, vol. 97, p. 19-24, (1993) discloses broadening of the surface plasmon line in a biosensor system in which an immunologic reaction of the antigen-antibody type takes place due to irregularities, roughness or even a discrete structure of the studied film, resulting in a sharp decrease of the sensitivity in the measurement of the reflectance curve minimum angle.

Salamon, Z., Wang, Y., Tollin, G. and Macleod, H., Biochimica et Biophysica Acta, 1195(2), p. 267-75 (1994) reports theoretical modelling for experimental surface plasmon resonance results to determine the thickness, refractive index, and extinction coefficient of self-assembled lipid bilayers, and to calculate the adsorbed mass and volume. From this calculated data the average steady state structure of the lipid layer is characterized. It is demonstrated that deposition of a lipid layer may change the SPR-reflectance curve so that (i) the position of the resonance minimum is shifted towards larger incident angles; (ii) the reflected light intensity at the resonance minimum is increased; and (iii) the SPR curve is broadened. The observation of only one resonance minimum indicates that the coverage of the silver surface of the sample molecules is homogeneous.

In summary, while as described above, it has been recognized in the prior art that the shape of the surface plasmon resonance curve may contain certain structural information on an SPR-probed solid or liquid film, measurement of the value (i.e. depth) of the reflectance minimum or the halfwidth of the reflectance curve have never been used in SPR-based biosensor applications for the monitoring of surface

interactions. Nor has it been suggested that such reflectance curve information would be of any use therein.

SUMMARY OF THE INVENTION

5 One object of the present invention is to provide in the analysis of interactions taking place at an optical sensor by surface refractive index measurement, such as SPR, means for determining when an interaction is mass transport limited and when it is kinetically
10 controlled.

Another object of the present invention is to provide in the analysis of an interaction taking place at an optical sensor by surface refractive index measurement, such as SPR, means for determining the degree of any
15 heterogeneity in the sensed surface layer during or resulting from the interaction.

According to the present invention it has been found that the above and other objects and advantages may be achieved by, in addition to monitoring the refractive index
20 variation at the surface by measuring the minimum, maximum or centroid of a light intensity signal curve, also monitoring the variation of the value of the light intensity signal at the minimum, maximum or centroid, and optionally also the halfwidth of the light intensity signal
25 curve.

More particularly, it has been found that a varying signal intensity minimum or maximum level will represent mass transport limitation of the interaction at the surface, whereas a stabilized minimum or maximum level
30 indicates kinetic control of the interaction. This will permit the kinetic constants to be measured more conveniently and with a substantially higher degree of accuracy than in the prior art methods.

Further, a changed (increased or decreased) stabilized
35 minimum or maximum level will indicate that there is a resulting homogeneity/heterogeneity change in the sensed film. Hereby, for example, also the time and degree of a

homogeneity/heterogeneity change in the sensed film may be accurately determined.

The present invention thus provides a method of analysing a chemical (including biochemical) or physical (including biophysical) interaction taking place in a film layer at an optical sensor surface when the film layer is contacted with a fluid sample containing a species capable of interacting with the film layer, wherein the interaction is monitored by determination of the refractive index of said film layer through a light-intensity signal producing technique by measuring the relationship between a parameter of the incident and/or reflected light and of one of the minimum, the maximum and the centroid of the light intensity signal curve. The method is characterized by, in addition to monitoring the refractive index, also monitoring the variation of the light intensity signal value at said minimum, maximum or centroid with time to determine at least one of (i) the degree of homogeneity of sample species concentration in the sensed film layer volume to determine therefrom when the interaction is mass transport limited and when the interaction is kinetically controlled, a varying light intensity signal level indicating mass transport limitation and a stabilized light intensity signal level indicating kinetic control; and (ii) the degree of homogeneity/heterogeneity in the sensed film layer volume during or after the interaction, a changed stabilized light intensity signal level indicating and corresponding to the degree of a change in homogeneity/heterogeneity.

The light-intensity signal producing technique used for measuring the refractive index may be selected from a variety of established optical methods, including conventional reflectometry, reflection ellipsometry, reflection interferometry and resonant guided mode detection in a waveguide. While these methods are based on the detection of a reflectance minimum, this minimum may also be manifested as a maximum in the light intensity

signal, e.g. by relating the reflected intensity from differently polarized light.

In conventional reflectometry, the sensor surface reflectivity (absolute value of reflection constants) of light is measured through the reflected light intensity as a function of a suitable parameter, usually angle of incidence and/or wavelength. The reflection mode may be internal (involving an evanescent wave), or external. Among internal reflection methods may be mentioned those based on surface plasmon resonance (SPR), Brewster angle, and critical angle. External reflection methods include Brewster angle detection.

In reflection ellipsometry, the phase shift (phase of reflection constants) introduced by the sensor surface on the polarization state of the reflected light is measured through the detected light intensity as a function of a suitable parameter, usually polarizer (analyser) angle, compensator angle, angle of incidence, or wavelength. The reflection mode may be internal (evanescent wave ellipsometry) or external (conventional ellipsometry).

In reflection interferometry, the phase shift (phase of reflection constants) introduced by the sensor surface and which changes the intensity or polarization state of the reflected light is measured through the detected light intensity as a function of a suitable parameter, usually polarizer (analyser) angle, compensator angle, angle of incidence, or wavelength. The reflection mode may be internal (involving an evanescent wave), or external.

In resonant guided mode detection in a waveguide, the mode coupling introduced by the sensor surface and which changes the transmission of the light via its absorption or phase shift is measured through the detected light intensity as a function of a suitable parameter, usually angle of incidence or wavelength. The reflection mode is internal (involving an evanescent wave). Methods using this detection technique include those based on frustrated total reflection (resonant cavity).

The light may interact with the sensor surface through transmission, grating-coupling, or coupling via frustrated total internal reflection (evanescent wave coupling), such as in resonant mirror devices.

5 The method of the invention may, for example, be performed with a combination of at least two wavelengths and a fixed incident angle. As another example, a combination of at least two wavelengths and at least two
10 incident angles may be used. In another example, a continuous scan of a predetermined incident angle or wavelength range is used. As still another example the monitoring may be performed using at least two different polarisation states and fixed incident angle and
15 wavelength. In another example, a continuous scan of a predetermined polarisation state range is used.

While the fluid sample may in principle be a gas, it is usually a liquid.

20 The method of the invention may, of course, be used in the monitoring of two or more sensor surfaces simultaneously as is per se known in the art.

25 One embodiment of the present invention comprises determining from the monitoring of the reflectance minimum or maximum value the time interval or intervals when the interaction at the surface is kinetically controlled, and
30 from the relationship between refractive index and time measured during this interval or intervals determining the association and/or dissociation constants for the interaction of the sample species with the surface layer. Preferably, such kinetic constant determination is
30 initiated automatically when it is determined that the interaction is kinetically controlled, for example, by modification of software used in the evaluation of the constants.

35 Another embodiment of the invention comprises determining from the monitoring of the reflectance minimum or maximum value the time interval when the interaction at the surface is mass transfer limited, and from the relationship between refractive index and time measured

during this interval or intervals determining the concentration of the sample species. Many times it is desirable to determine both concentration and a kinetic constant or constants.

5 The homogeneity/heterogeneity of the film layer usually relates to one or more of the physical condition (concentration structure, protein aggregates, crystallinity, phase, gas), the thermodynamic condition (temperature, surface energy) and the chemical condition
10 (pH, ionic strength, charge) of the film layer.

Two different cases may be identified depending on the size of the sample (analyte) molecule:

(i) A macromolecular sample in solution (e.g. protein) binds to/reacts with the immobilized film which is
15 activated and thereby changed to be more heterogeneous or more homogeneous. In the first case, the reflectance minimum is stabilized at a higher level than before the sample addition, whereas in the latter case the reflectance minimum is stabilized at a lower level, as will be
20 described in more detail below. Homogeneity of the sample binding will then have to be detected on a reference sensor surface. Thereby, a measured and quantified homogeneity change may be attributed to the immobilized film.

(ii) A low-molecular sample in solution (e.g. drugs, small molecules produced by cells in solution outside the
25 sensor volume) binds to/reacts with the immobilized film which is activated and thereby changed to be more heterogeneous or more homogeneous. These small molecules are, however, not capable of giving any significant
30 refractive index change by themselves, but may activate a significant structural change of a macromolecular immobilized film.

Often it may be valuable to determine the sample species concentration related to a particular change in
35 homogeneity/heterogeneity of the film layer. Determination of homogeneity/heterogeneity may also be used as a quality control of a pre-immobilized reactant concentration or structure with regard to homogeneity.

Particularly, heterogeneity in the surface film layer is selected from protein aggregates caused by precipitation, agglutination or flocculation reactions; membrane complexes, such as biological cells and cell
5 membrane fragments; macromolecular product complexes caused by enzymatic reactions; and cellular product complexes.

A stability test of a protein solution may be performed by simultaneously determining bound protein concentration and heterogeneity in the bound protein film
10 due to protein aggregates in the sample.

When the heterogeneity consists of membrane complexes, a simultaneous determination may be made of bound sample concentration and related binding or release of particulate sample, such as biological cells or cell membrane
15 fragments.

When the heterogeneity consists of macromolecular product complexes, a simultaneous determination may be made of bound sample concentration and related enzymatic production, activation or inhibition, such as coagulation, fouling, enzymatic cascade reactions (such as in the blood
20 coagulation system or the immunological complement system).

When the heterogeneity consists of cellular adhesion distribution or cellular product complexes, a simultaneous determination may be made of either sensor surface bound
25 sample concentration or solution concentration, and related cellular adhesion distribution, like proliferation or degradation, or cellular molecule production.

Monitoring of also the halfwidth of the light signal intensity curve in question may provide additional
30 information on the state of homogeneity/heterogeneity within the sensed surface volume.

In the case of an internal reflection based method, data measured at a number of wavelengths may be used to determine the state of homogeneity/heterogeneity in the
35 direction of the penetration depth of the evanescent wave, the penetration depth varying with wavelength.

In the following the invention will be described in more detail primarily with regard to SPR detection, reference being made to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Fig. 1 shows (i) a schematic overlay plot of a sensorgram (SPR response vs time) and a reflectance minimum vs time curve for SPR measurements of surface interactions, and (ii) a number of schematic sectional views of a flow cell illustrating different states in the flow cell
10 corresponding to defined parts of the sensorgram and the reflectance minimum curves;

 Fig. 2a is an overlay plot of a sensorgram (SPR response vs time) and a reflectance minimum vs time curve for the interaction of trypsin with immobilized trypsin
15 inhibitor;

 Fig. 2b is a plot of calculated reflectance vs angle of incidence curves for three different refractive indices for a two-phase film model corresponding to the interaction in Fig. 2a;

20 Fig. 3a is an overlay plot of a sensorgram (SPR response vs time) and a reflectance minimum vs time curve for the adsorption of rabbit antimouse IgG;

 Fig. 3b is a plot of calculated reflectance vs angle of incidence curves for three different refractive indices
25 for a two-phase film model corresponding to the interaction in Fig. 3a;

 Fig. 4a is a similar overlay plot to that of Fig. 3a of a sensorgram (SPR response vs time) and a reflectance minimum vs time curve for the interaction of monoclonal
30 antibody with immobilized rabbit antimouse IgG but where the interaction causes a permanent heterogeneity in the sensing surface due to aggregate formation;

 Fig. 4b is a plot of calculated reflectance vs angle of incidence curves for four different refractive indices
35 for a two-phase film model corresponding to the interaction in Fig. 4a;

 Fig. 4c is a plot of calculated reflectance vs angle of incidence curves for four different refractive indices

for a three-phase film model corresponding to the interaction in Fig. 4a.

DETAILED DESCRIPTION OF THE INVENTION

In Fig. 1 is illustrated schematically in overlay
5 format a typical SPR derived reflectance curve (SPR response, or resonance angle, versus time curve), or sensorgram, and a corresponding reflectance minimum (R_{\min}) curve for the interaction of an analyte-containing sample solution with surface-immobilized reactant in a flow-cell,
10 e.g. as obtained with a BIAcore™ system (Pharmacia Biosensor AB, Uppsala, Sweden). The analyte is, for example, an antibody and the immobilized reactant an antigen, or vice versa.

Below the curve diagram are shown a number of
15 schematic cross-sections of the flow-cell, each designated by a numeral and representing different phases of the passage of the sample through the flow cell. The point on the sensorgram (SPR angle) and reflectance minimum (R_{\min}) curve, respectively, that corresponds to a respective flow
20 cell section is indicated by the same numeral as the flow cell section. The monitored sensing surface volume (containing the reactant), or sensor volume, which corresponds to the penetration depth of the evanescent wave, say $\approx 0.7 \mu\text{m}$, is designated by the letter S in the
25 flow cell sections. The bottom part of the sensor volume contains a reaction layer, or matrix, (e.g. with a depth of about 100 nm) where the reactants are immobilized (not shown).

Numeral 1 designates the base line of the two curves,
30 corresponding to the flow cell state prior to the introduction of the sample solution and with only solvent passing through the cell. The refractive index of the sensor volume, which for illustration may be said to be 1.335, is defined by the refractive indices of the solvent
35 and the immobilized reactant.

At 2 (time=0) sample solution (solvent L plus dissolved sample P) starts entering the flow cell, no change of refractive index yet taking place.

At 3 the sample front has almost passed through the flow cell section and there is a high sample heterogeneity within the flow cell volume. The refractive index is still mainly determined by solvent and immobilized reactant, as
5 the sample analyte is concentrated to the central core of the flow and has not diffused to the sensor volume to any great extent. While the refractive index starts to increase at this point, there is no notable effect on the reflectance minimum value.

10 At 4 ($t=t_1$) the flow cell is completely filled by sample solution (i.e. homogeneity with respect to sample solution), and analyte starts diffusing into the reaction layer and bind to the immobilized reactant. The reaction kinetics is mass transport limited, permitting the sample
15 concentration within the sensor volume to be determined from this part of the reflectance curve as is well established in the prior art. The refractive index at this point may for illustration be e.g. about 1.336.

At 5 about half of the lateral extension of the sensor
20 volume now contains bound reactant, resulting in a maximum heterogeneity of the sensor volume, which in turn causes a peak of the reflectance minimum value. At this stage the kinetics is dependent on both mass transport and reaction rate.

25 At 6 reactant has bound in the whole lateral extension of the sensor volume, and the analyte concentration within the sensor volume is homogeneous. The reflectance minimum value has decreased to base-line level. The reaction kinetics is now totally controlled by the reaction rate and
30 the association rate constant may be determined from the sensorgram. The refractive index at this point is about 1.356.

At 7 ($t=0$) the whole sample plug has almost passed the flow cell and starts being replaced by wash solution L
35 displacing unbound analyte.

At 8 the wash solution fills more than half the flow cell volume, and unbound or loosely bound analyte has been removed from about half sensor volume. This results in an

instant heterogeneity in the sensor volume which causes the fast spikes observed in the reflectance minimum curve. The corresponding step in the sensorgram curve is caused by the bulk refractive index change. The refractive index at this point may e.g. for illustration be about 1.355.

At 9 ($t=t_1$ from the introduction of wash solution) there is a homogeneous reactant concentration within the sensor volume, and the reflectance minimum curve therefore returns to substantially the same baseline level as before the sample plug. Kinetically controlled dissociation of bound analyte has started and the dissociation rate constant may be calculated.

10 represents the corresponding point as 9 above for the case of a resulting higher degree of (permanent) heterogeneity within the sensor volume. The reflectance minimum curve does therefore not return to the original baseline level as at 9 but exhibits a raised baseline level. As at 9 above, the dissociation is reaction limited and the dissociation rate constant may be calculated.

At 11 (no flow cell section shown) there is significant desorption in case of a high dissociation constant, resulting in a high sample heterogeneity within the sensor volume, which in turn causes a peak of the reflectance minimum curve. At this stage the kinetics is dependent on both mass transport and reaction rate. The sensorgram curve has now reached a lower level, corresponding to a refractive index of say about 1.350.

For an exemplary flow cell volume of $0.05 \mu\text{l}$ and a flow rate of $5 \mu\text{l}/\text{min.}$, the time t_1 when the liquid in the flow cell has been exchanged will be 0.6 seconds.

As demonstrated above, monitoring of the reflectance minimum (R_{\min}) curve will indicate (i) when sample reaches the sensor surface and starts binding thereto, and (ii) when the sample plug has passed and is replaced by wash solution displacing sample. This applies both to the injection of sample into a carrier buffer flow and for segmented sample solution transport. Thus, it is possible to determine the time of simultaneous presence of buffer in

a part of the sensed surface and of sample in another part of the sensed surface, and to relate this time to a corresponding SPR angle change.

This permits the measurement of kinetic constants to
5 be repeatably synchronized to the time when the association and/or dissociation phases, respectively, are reaction rate controlled, that is when R_{\min} has passed the peak and is stabilized again. In an instrument like, for example, the above-mentioned BIAcore™ (Pharmacia Biosensor AB), means
10 (e.g. software-based) may readily be designed to automatically provide for such synchronisation.

It is understood that the achieved increased accuracy in the determination of the above times for kinetic control of the association and dissociation phases will permit the
15 kinetic constants to be determined with a considerably higher accuracy than before and with substantially less labour and trouble.

It has also been demonstrated above that simultaneously with the kinetics of SPR angle data, the
20 degree of heterogeneity within the reactant film may be determined, for example as the result of the formation or dissolution of aggregates, crystallization, gas formation, temperature changes, phase or domain formation/resolution.

Thereby, for example, the time, specificity or surface
25 concentration of the start of a homogeneity/heterogeneity change in the structure or state of the reactant film may be accurately determined, preferably automatically as above. Via the SPR angle-time curve, the corresponding specificity or surface concentration of the reactant film
30 may be determined.

By such automatic measurement of the time, specificity and/or surface concentration for a quantified change of the homogeneity/heterogeneity within the volume sensed by the evanescent wave, the dependence of this reaction on the
35 functional characteristics of a sample molecule may be determined with considerably higher accuracy than before and with less labour and trouble.

Of course, also the presence of permanent changes in the sensor film structure may readily be determined.

The experiments presented in the following-non-limiting examples were performed with a BIAcore™ system, using a sensor chip CM5 (a gold-plated glass slide supporting a carboxymethyldextran layer via a monolayer of long-chain hydrocarbons), both supplied by Pharmacia Biosensor AB, Uppsala, Sweden.

EXAMPLE 1

Soybean trypsin inhibitor (STI) (Sigma) was immobilized to the sensor chip surface according to the manufacturer's instructions. The surface was first activated with 1:1 N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for 3 minutes. 80 µg/ml of STI in 10 mM citric acid, pH 4.0, were then injected for 7 minutes. Deactivation of remaining activated groups was performed with ethanolamine-hydrochloride, pH 8.5, for 7 minutes. The surface was washed with HCl. The volumetric flow during the immobilization procedure was 5 µl/min. As buffer was used 10 mM Hepes, 150 mM NaCl and 0.05 % BIAcore™ Surfactant P20, pH 7.4.

Kinetic analysis procedures with different concentrations of bovine pancreatic trypsin (BTP) (Sigma) were then run to determine kinetic constants for the interaction of BTP with the immobilized STI. The volumetric flow rate was 10 µl/min.

The SPR response and the reflectance minimum, R_{\min} , were monitored in the experiments. The point of time corresponding to the maximum value of R_{\min} , $t(R_{\min})$, was extracted from the reflectance minimum curve. The sampling time was 0.2 seconds. Regeneration of the sensor surface was performed with HCl.

Fig. 2a shows a typical sensorgram part with associated R_{\min} curve, as obtained for 25 nM trypsin and a surface concentration of STI corresponding to 413 RU (Resonance Units - 1000 RU corresponds to a 0.1° shift in SPR angle, or a refractive index change of 0.001). As

appears from Fig. 2a, a maximum of the R_{\min} curve occurred during the association part (the illustrated part of the sensorgram). The R_{\min} change is about 0.06% coinciding with about 60 % of the maximum change of the R_{\min} angle. R_{\min} then returns to approximately the level for sample-free solution.

The above case represents the situation of two phases having close refractive indices/reflectance curves. A calculation of R_{\min} for a two-phase film model with a transition via a coverage 0.4 of phase 1, $n=1.335$, and coverage 0.6 of phase 2, $n=1.3353$, gave the results shown in Fig. 2b. The R_{\min} peak value was about 0.02%, coinciding with about 58% of the maximum change of the R_{\min} angle corresponding to a coverage 1.0 of phase 2.

From the analyses data it could be determined that the occurrence of the mass transfer/kinetically controlled break point correlated to the time event when R_{\min} had passed the maximum value and returned to a stabilized level. By monitoring the R_{\min} value together with the sensorgram the time value corresponding to the boarder between mass transfer controlled and kinetically controlled interaction may thus be determined. Feeding this time value into the evaluation scheme will therefore have the potential to simplify and improve the association and dissociation rate constants calculation.

EXAMPLE 2

In analogous manner to Examples 1 and 2, the immobilization of rabbit antimouse IgG (RAMFc) onto a sensor surface by adsorption was studied at a flow rate of 5 $\mu\text{l}/\text{min}$. The sensorgram and the reflectance minimum (R_{\min}) curve obtained are shown in Fig. 3a. As appears from the figure, the peak of the R_{\min} value is about 26% (15611/60000) and coincides with about 70% of the maximum change of the R_{\min} angle. After the peak, R_{\min} returns to approximately the value for the sample-free solution.

The above case represents the situation of a high sample concentration and two phases with well separated refractive index/reflectance curves. A calculation of R_{\min}

for a two-phase film model with a transition via a coverage 0.57 of phase 1, $n=1.3339$, and coverage 0.43 of phase 2, $n=1.3498$, gave the results shown in Fig. 3b. The R_{\min} peak value was about 20%, coinciding with about 57% of the maximum change of the R_{\min} angle.

EXAMPLE 3

In analogous manner to Example 1, a sensor surface was immobilized with rabbit antimouse IgG (RAMFc) of another batch and the interaction with the surface was studied at a flow rate of 5 $\mu\text{l}/\text{min}$. The sensorgram and the reflectance minimum (R_{\min}) curve obtained are shown in Fig. 4a. As appears from the figure, the peak of the R_{\min} value is about 28% (16900/60000) and coincides with about 74 % of the maximum change of the R_{\min} angle. After the peak, R_{\min} does not return to the value for the sample-free solution but is stabilized at a higher level, indicating a permanent heterogeneity within the sensed surface layer volume. Particle precipitation (RAMFc aggregate formation) in the sample solution was also detected by a separate independent method.

The above case represents the situation of a high sample concentration and particle precipitation, two phases with well separated refractive index/reflectance curves and a particulate surface film structure. A calculation of the corresponding R_{\min} for a two-phase film model with a transition via a coverage 0.6 of phase 1, $n=1.3425$, and a coverage 0.4 of phase 2, $n=1.3512$, gave the results shown in Fig. 4b. The R_{\min} peak value was about 23%, coinciding with about 79% of the maximum change of the R_{\min} angle at a coverage 1 of phase 2.

A corresponding calculation of R_{\min} for a three-phase film with a transition via a coverage 0.33 of phase 1, $n=1.3339$, a coverage 0.33 of phase 2, $n=1.3425$, and a coverage 0.33 of phase 3, $n=1.3512$, gave the results shown in Fig. 4c. The R_{\min} peak value was about 35%, coinciding with about 82% of the maximum change of the R_{\min} angle.

While none of the above two calculation models gave results corresponding to the experimental data, they

correspond sufficiently well to demonstrate that it is possible to fit a greater number of phases of respective refractive indices as well as a distribution of surface coverage to simulate experimental results.

CLAIMS

1. A method of analysing a chemical or physical interaction taking place in a film layer at an optical sensor surface when the film layer is contacted with a fluid sample containing a species capable of interacting with the film layer, wherein the interaction is monitored by determination of the refractive index of said film layer through a light-intensity signal producing technique by measuring the relationship between a parameter of the incident and/or reflected light and one of the minimum, the maximum and the centroid of the light intensity signal curve, characterized by also monitoring the variation of the light intensity signal value at said minimum, maximum or centroid with time to determine at least one of (i) the degree of homogeneity of sample species concentration in the sensed film layer volume to determine therefrom when the interaction is mass transport limited and when the interaction is kinetically controlled, a varying light intensity signal level indicating mass transport limitation and a stabilized light intensity signal level indicating kinetic control; and (ii) the degree of resulting homogeneity/heterogeneity in the sensed film layer volume during or after the interaction, a changed stabilized light intensity signal level indicating and corresponding to the degree of a change in homogeneity/heterogeneity.
2. The method according to claim 1, characterized in that said fluid sample is a liquid.
3. The method according to claim 1 or 2, characterized by determining from said monitoring of the light intensity signal value the time interval or intervals when said interaction is kinetically controlled, and from the relationship between refractive index and time measured during said interval or intervals determining the association and/or dissociation constants for the interaction of the sample species with the surface layer.

4. The method according to claim 3, **characterized** by automatically initiating said determination of the association and/or dissociation constants when the
5 interaction is kinetically controlled.

5. The method according to claim 3, **characterized** by determining from said monitoring of the light intensity signal value the time interval or intervals when said
10 interaction or interactions are mass transfer limited, and from the relationship between refractive index and time measured during said interval or intervals determining the concentration of the sample species.

15 6. The method according to claims 3 or 4 and 5, **characterized** by determining both sample species concentration and association and/or dissociation constants.

20 7. The method according to any one of claims 1 to 6, **characterized** in that said homogeneity/heterogeneity relates to one or more of the physical condition, the thermodynamic condition and the chemical condition of said film layer.

25 8. The method according to claim 7, **characterized** in that said homogeneity/heterogeneity in the surface film layer is selected from protein aggregates caused by precipitation, agglutination or flocculation reactions; membrane
30 complexes, such as biological cells and cell membrane fragments; macromolecular product complexes caused by enzymatic reactions; and cellular product complexes.

9. The method according to claim 8, **characterized** by
35 additionally measuring the sample species concentration related to the measured homogeneity/heterogeneity.

10. The method according to claim 9, **characterized** by pre-immobilizing a reactant ligand to the sensor surface film layer and determining any heterogeneity in the resulting ligand concentration or structure therein.
- 5
11. The method according to any one of claims 1 to 10, **characterized** by additionally monitoring the halfwidth of the light intensity signal curve versus time to obtain additional information on the degree of resulting
- 10 homogeneity/heterogeneity within the sensed film volume.
12. The method according to any one of claims 1 to 11, **characterized** in that said light intensity producing signal technique is based on reflectometry, reflection
- 15 ellipsometry, reflection interferometry or resonant guided mode detection in a waveguide.
13. The method according to claim 12, **characterized** in that said technique is based on internal reflection,
- 20 including measurement of surface plasmon resonance, Brewster angle, critical angle, ellipsometry or resonant mode coupling, or is based on external reflection, including measurement of Brewster angle or ellipsometry.
- 25 14. The method according to any one of claims 1 to 13, **characterized** in that said parameter of the light is selected from wavelength, incident angle and polarisation state.
- 30 15. The method according to claim 14, **characterized** by monitoring said interaction using at least two different wavelengths and a fixed incident angle.
16. The method according to claim 14, **characterized** by
- 35 monitoring said interaction using at least two different wavelengths and at least two different incident angles.

17. The method according to claim 14, **characterized** by monitoring said interaction using a continuous scan of a predetermined incident angle or wavelength range.

5 18. The method according to claim 14, **characterized** by monitoring said interaction using at least two different polarisation states and a fixed incident angle and wavelength.

10 19. The method according to claim 14, **characterized** by monitoring said interaction using a continuous scan of a predetermined polarisation state range.

15 20. The method according to claim 13, **characterized** by using internal reflection data measured at a number of wavelengths to determine the state of homogeneity/heterogeneity in the direction of the penetration depth of the evanescent wave, said penetration depth varying with wavelength.

20 21. The method according to any one of the preceding claims, **characterized** by analysing interactions at two or more sensor surfaces simultaneously using a single light beam.

25 22. The method according to any one of the preceding claims, **characterized** in that said fluid sample is contacted with the film layer in a liquid flow, preferably in a flow cell.

30 23. The method according to any one of the preceding claims, wherein a parameter of the incident and/or reflected light and one of the minimum and the centroid of the light intensity signal curve are measured,
35 **characterized** by also monitoring the variation of the light intensity signal minimum value with time.

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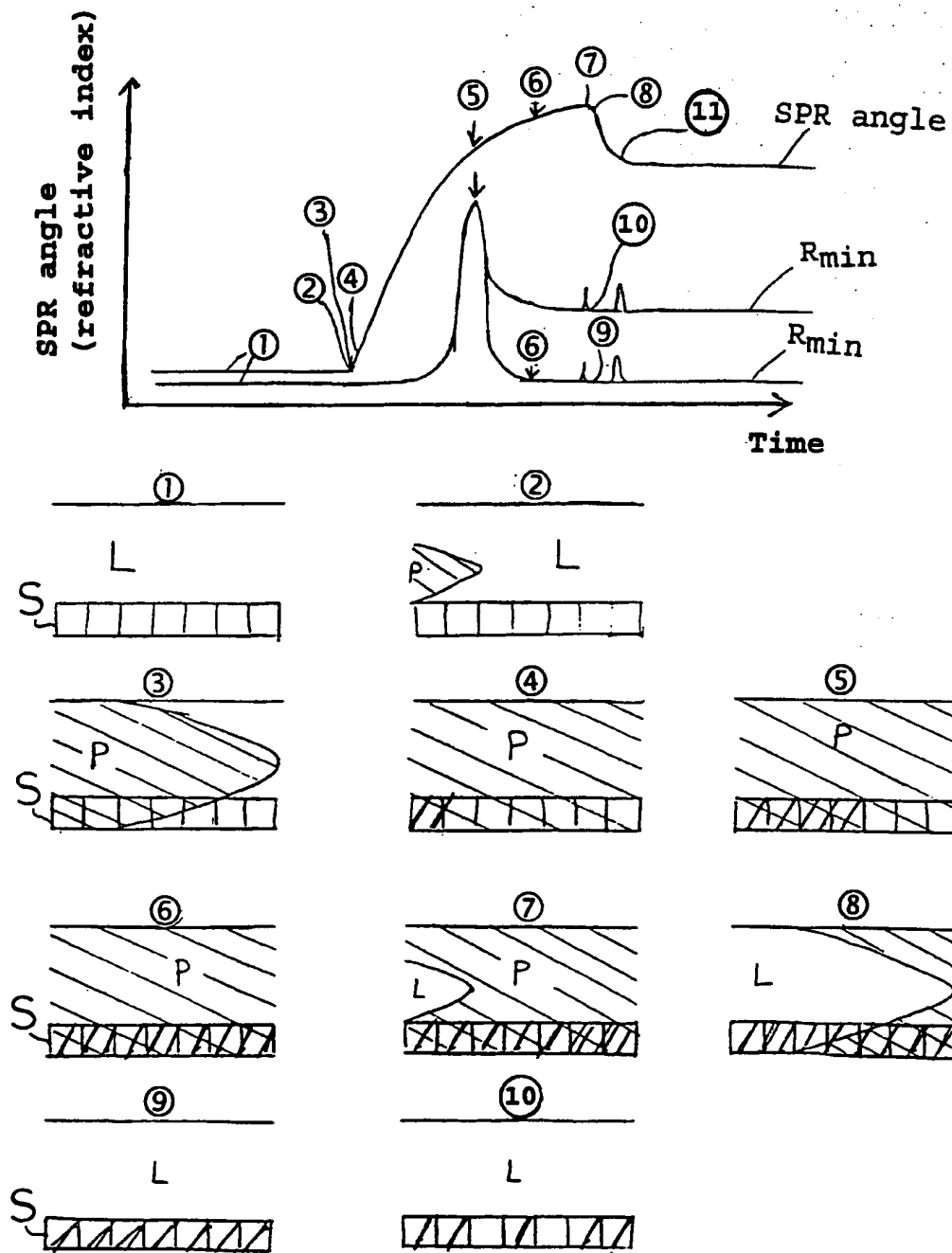


FIG. 1

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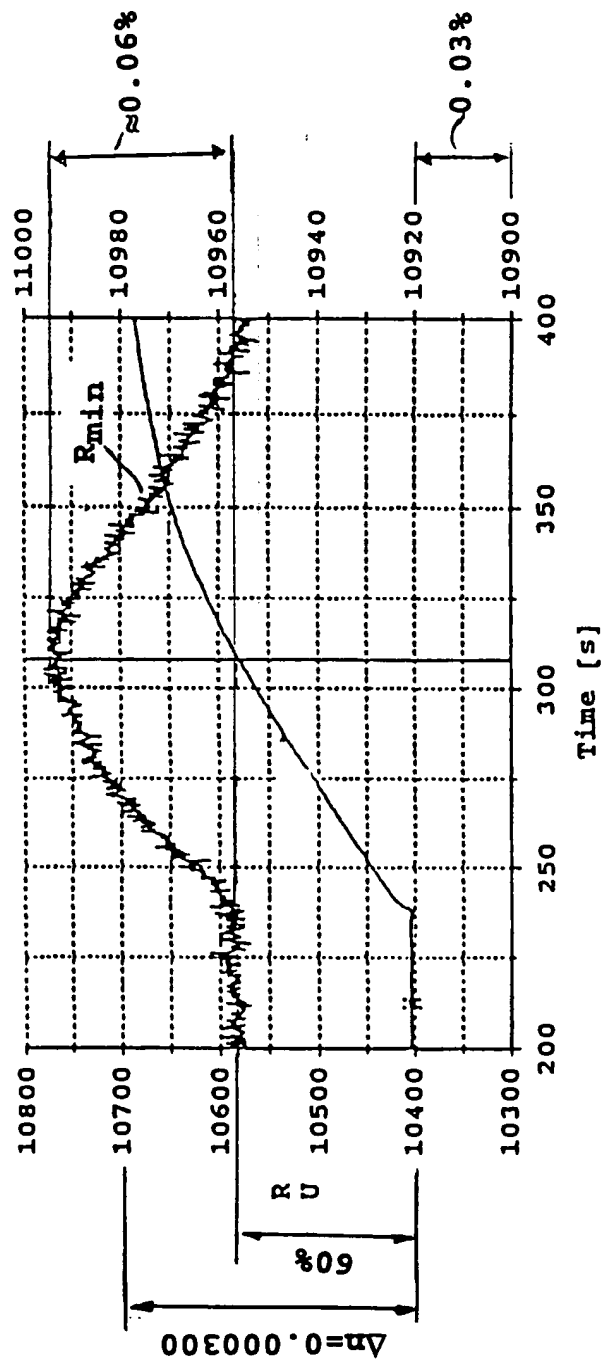


FIG. 2a

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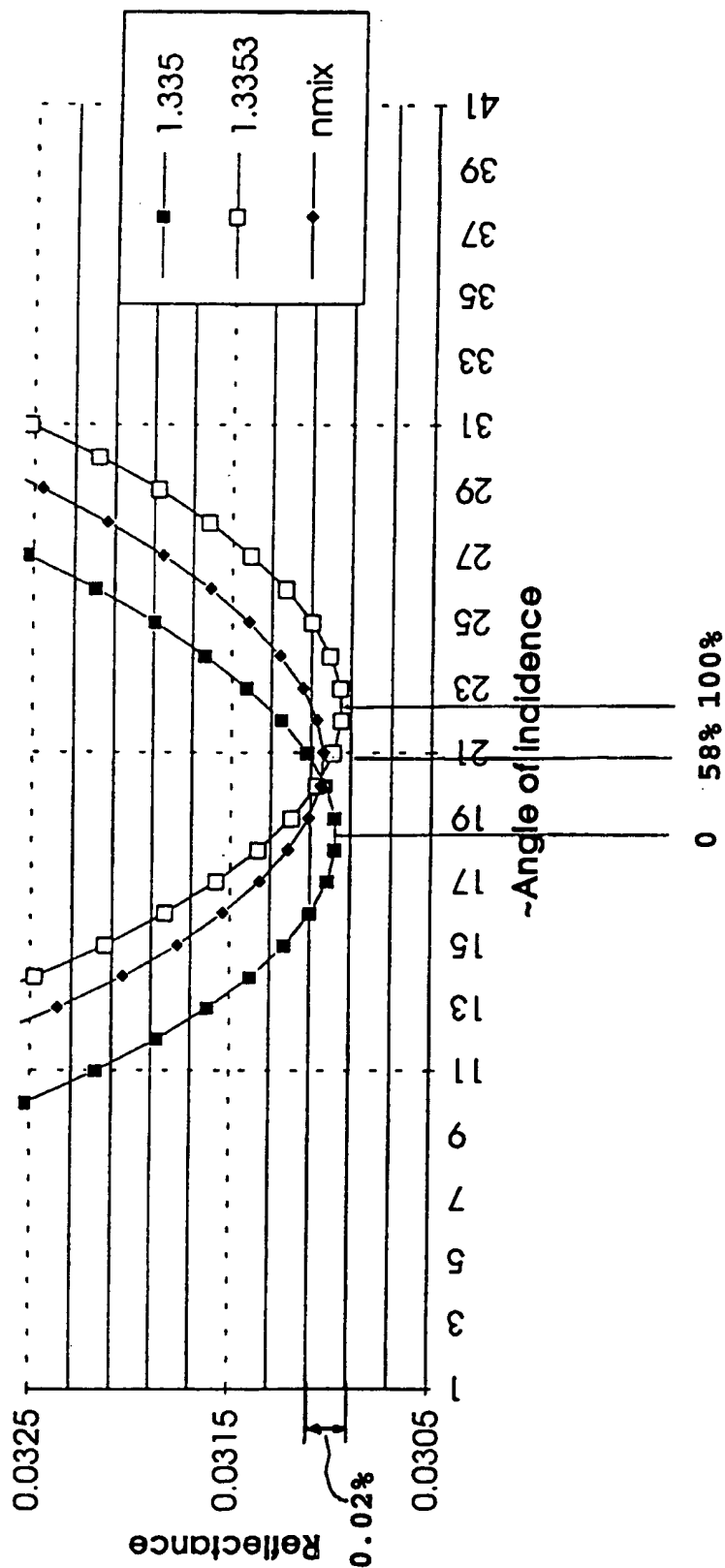


FIG. 2b

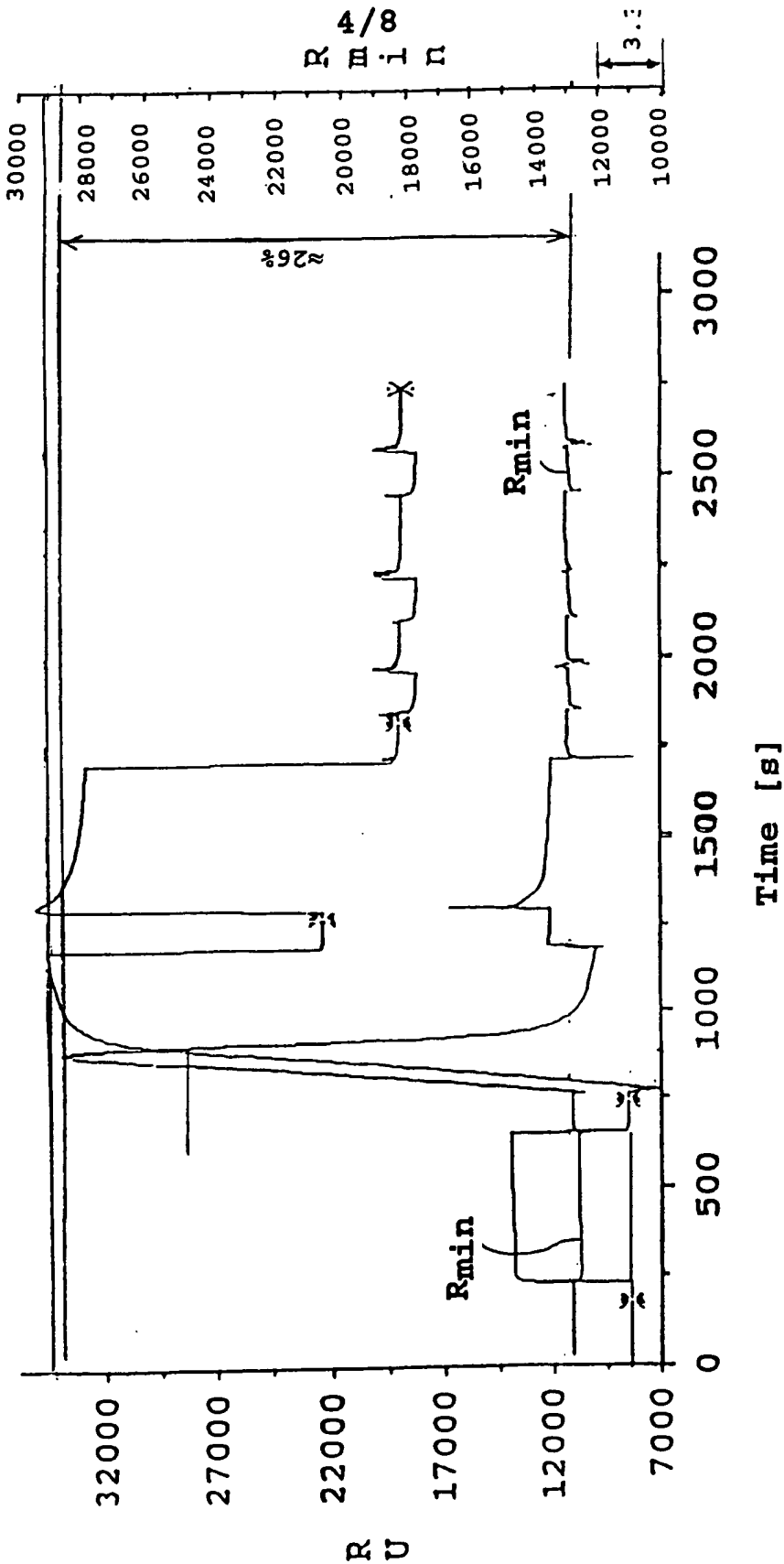


FIG. 3a

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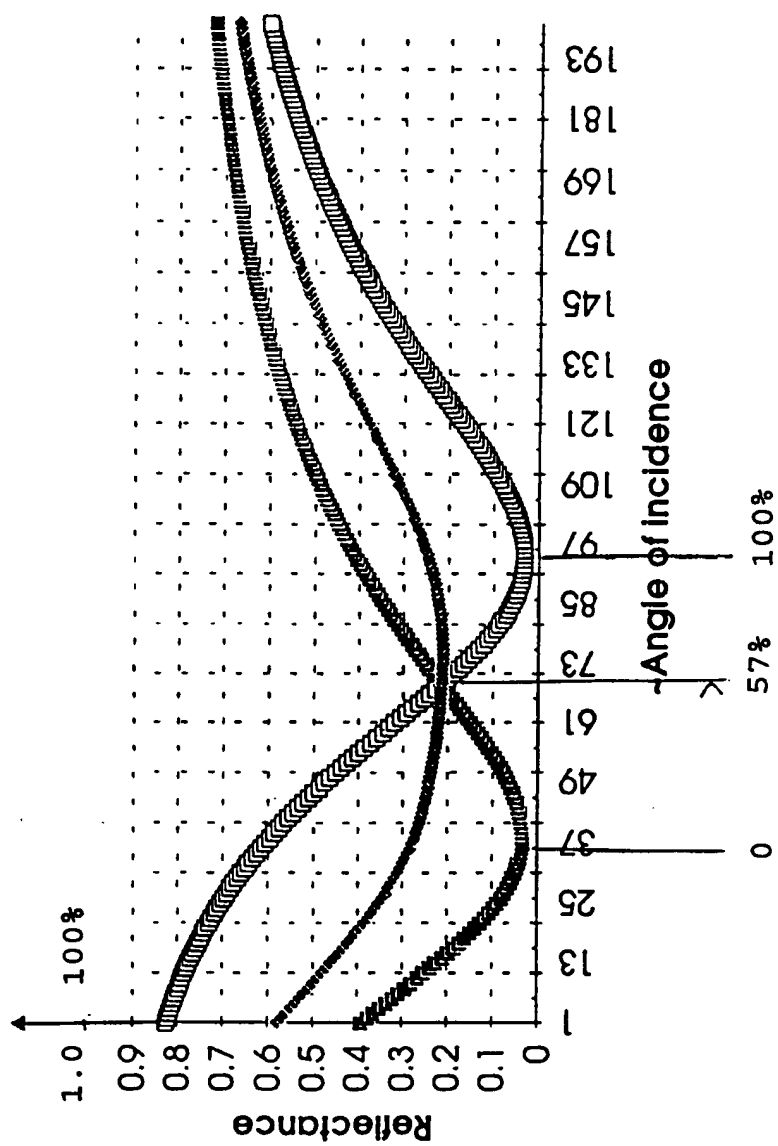


FIG. 3b

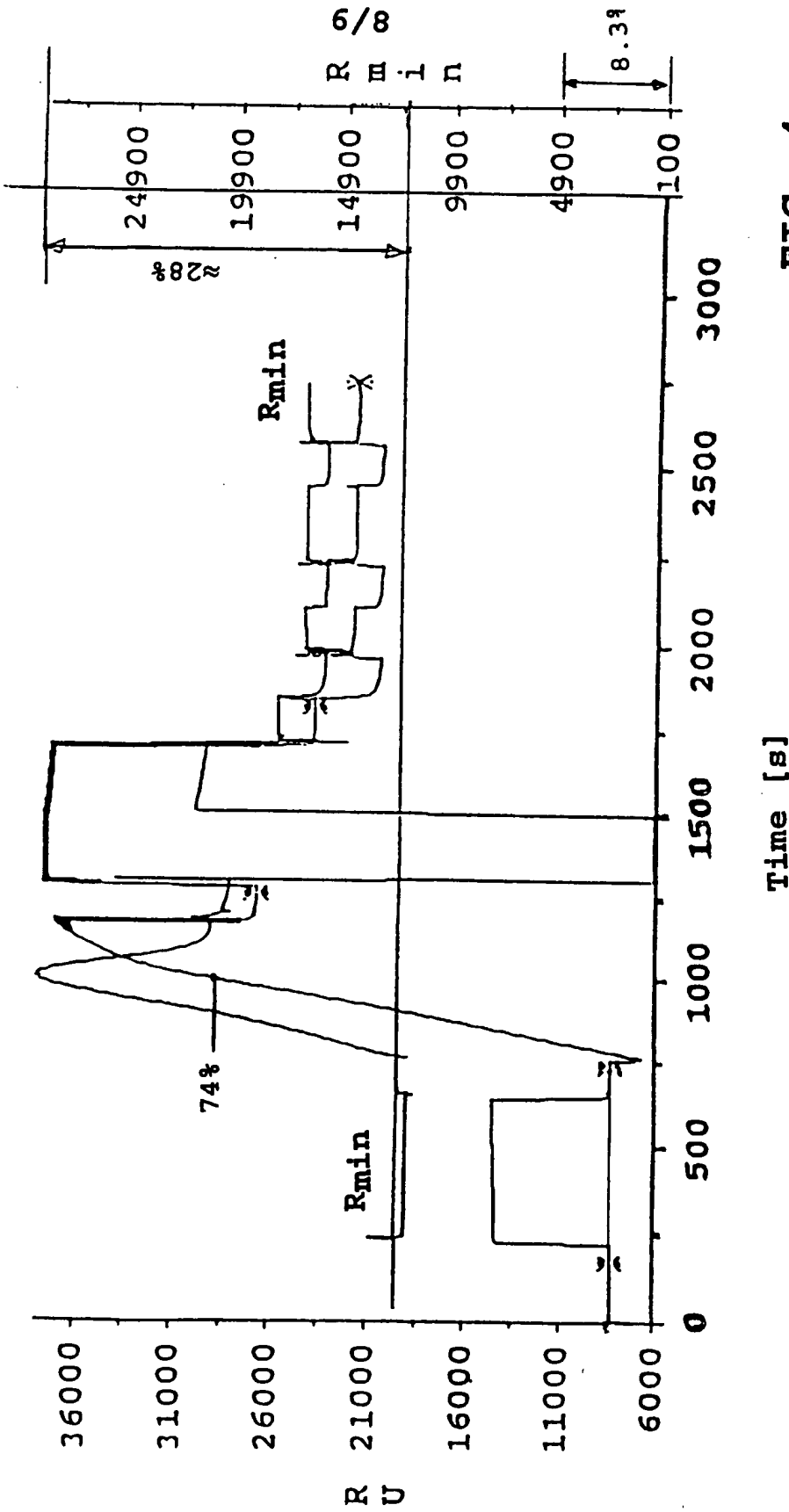


FIG. 4a

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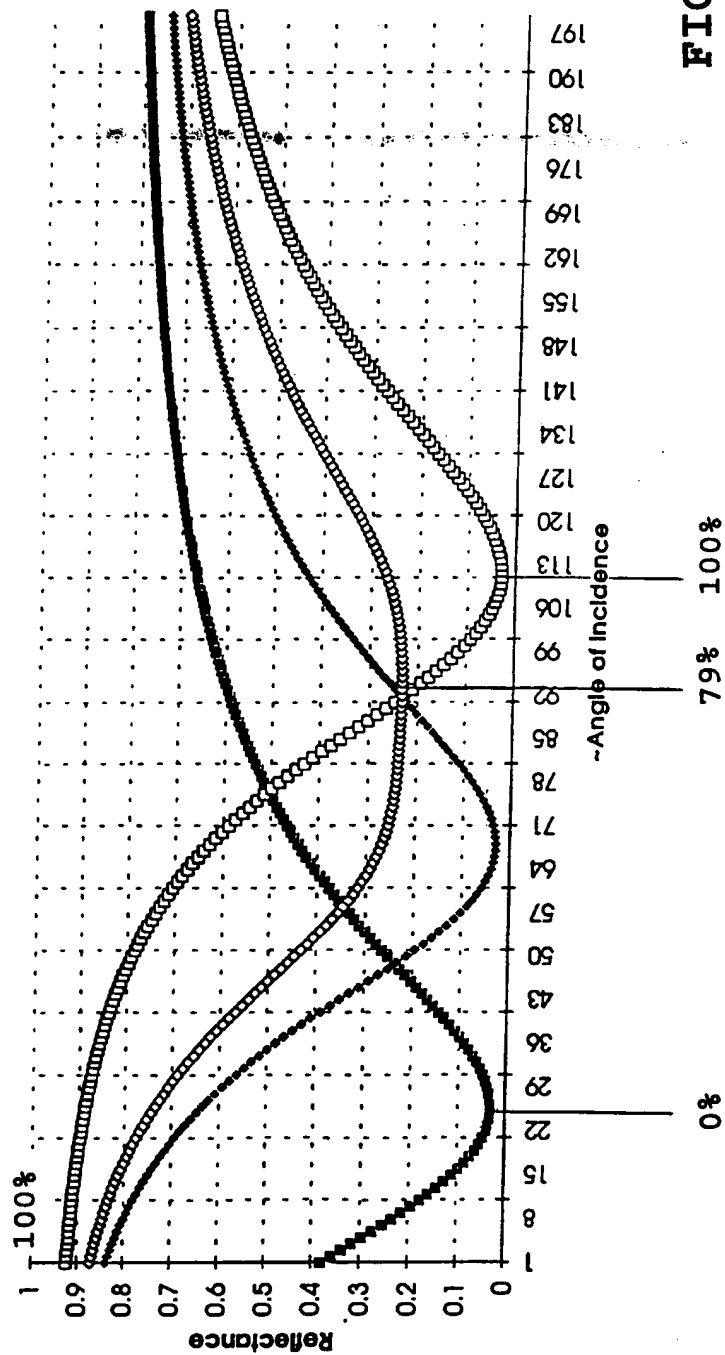


FIG. 4b

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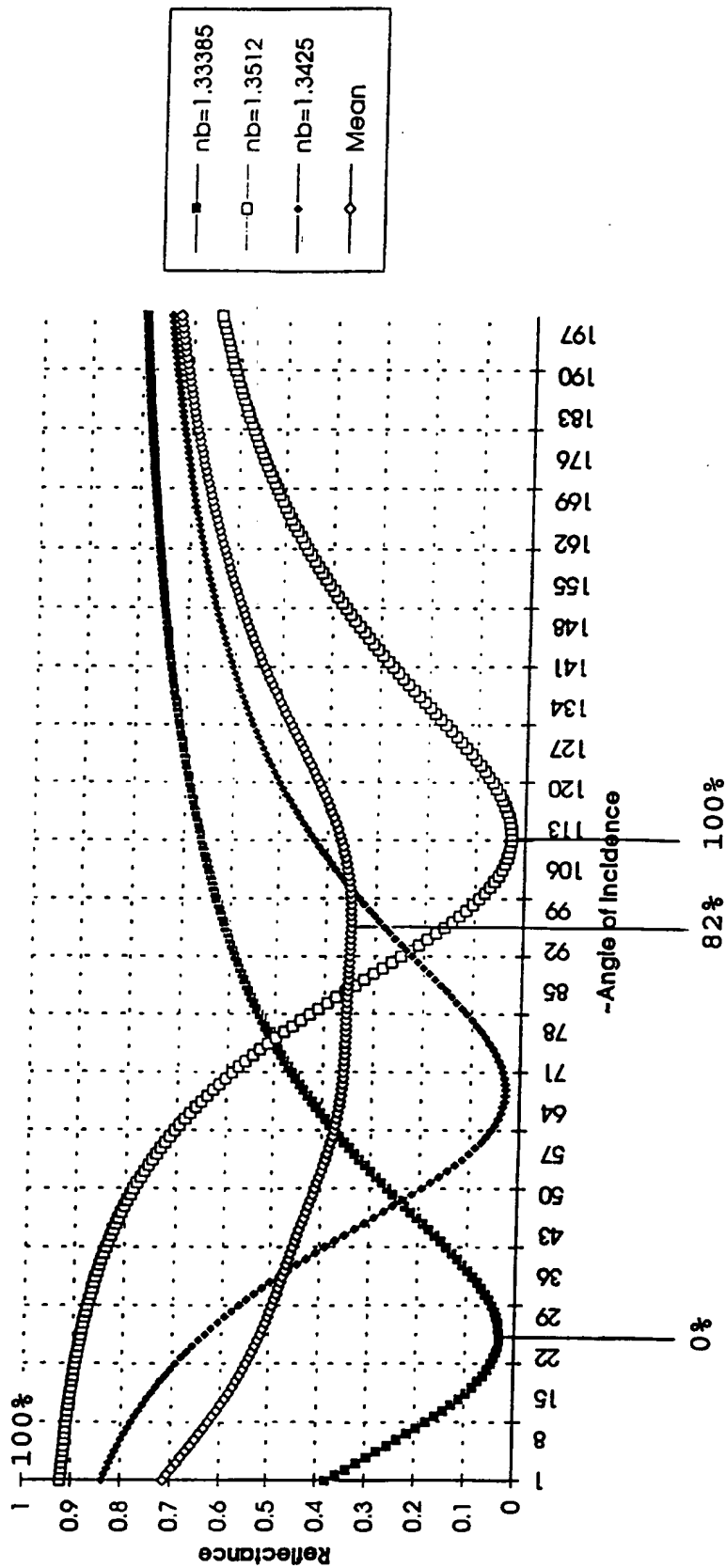


FIG. 4c

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01074

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 21/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Chromatography, Volume 597, 1992, Lars G. Fägerstam et al, "Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis", page 397 - page 410, see pages 401, 404-406, figures 12,13 and 20 --	1-3,5-10, 12-22
X	Analytical Biochemistry, Volume 213, 1993, Ralf W. Glaser, "Antigen-Antibody Binding and Mass Transport by Convection and Diffusion to a Surface: A Two-Dimensional Computer Model of Binding and Dissociation Kinetics", page 152 - page 161, see pages 152-3 and 160 --	1-3,5-10, 12-22

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

10 December 1996

12 -12- 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01074

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Analytical Biochemistry, Volume 227, 1995, Thomas A. Morton et al, "Interpreting Complex Binding Kinetics from Optical Biosensors: A Comparison of Analysis by Linearization, the Integrated Rate Equation, and Numerical Integration", page 176 - page 185, see page, the whole document eg. page 183, right column second paragraph</p> <p>--</p>	1-22
A	<p>Eur.J. Biochem., Volume 217, 1993, L. Clem Gruen et al, "Determination of relative binding affinity of influenza virus N9 sialidases with the Fab fragment of monoclonal antibody NC41 using biosensor technology", page 319 - page 325, see figures 1-3</p> <p>--</p>	1-22
A	<p>Biochimica et Biophysica Acta, Volume 1195, 1994, Zdzislaw Salamon et al, "Assembly and molecular organization of self-assembled lipid bilayers on solid substrates monitored by surface plasmon resonance spectroscopy" page 267 - page 275</p> <p>--</p>	1-22
A	<p>Optics Communications, Volume 64, No 6, December 1987, J.D. Pollard et al, "The Time-Dependence of the Growth of Thin Organic Liquid Layers on a Gold Surface, Studied by Surface-Plasmon Polariton Techniques" page 529-533</p> <p>--</p> <p>-----</p>	1-22

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